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Epidemiology of Enterovirus 71 in The Netherlands, 1963 to 2008▽

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The incidence of enterovirus 71 (EV71) infection has greatly increased in the Asian Pacific region since 1997. Several large outbreaks, caused by different subgenogroups of EV71, occurred with high rates of morbidity and a substantial number of deaths. In 2007, 58 cases of EV71 infection requiring hospitalization were reported in The Netherlands after a period of low endemicity of 21 years. These events triggered a study on the epidemiology of EV71 in The Netherlands. Genetic analysis of the VP1 capsid region of 199 EV71 isolates collected from 1963 to 2008 as part of enterovirus surveillance activities revealed a change in the prevailing subgenogroups over time. From 1963 to 1986 infections were caused by three different and successive lineages belonging to subgenogroup B (the novel lineage designated B0, as well as B1 and B2). In 1987, following a major epidemic the previous year, the B genogroup was replaced by genogroup C strains of lineages C1 and, later, C2. Analyses of the clinical data suggested that there were differences between infection with genogroup B and with genogroup C strains in terms of the age groups affected and the severity of illness. From comparative analysis with genomic data available in the public domain, we concluded that EV71 strain evolution shows a global pattern, which leads to the question of whether the recently emerged C4 lineage strains will also spread outside of Asia.

The genus *Enterovirus* of the family *Picornaviridae* consists of nine species of which five infect humans: poliovirus and human enterovirus A, B, C, and D. Together with 11 serotypes of coxsackievirus A, human enterovirus 71 (EV71) belongs to the human enterovirus A species. Human EV71 was first isolated in 1969, and from that time onward outbreaks of this virus have been described globally (38). On the basis of VP1 nucleotide sequence comparisons, three genogroups have been distinguished: A, B, and C (4, 29). Genogroup A includes only one strain (BrCr-CA-70) isolated in California in 1970 (4). Genogroup B is more common and consists of the previously defined subgenogroups B1 to B5, and genogroup C consists of subgenogroups C1 to C5 (4). Together with its close relative coxsackievirus A16, EV71 is the major causative agent of hand, foot, and mouth disease (HFMD) (usually in children of <5 years of age). EV71, however, is also associated with neurologic disease, including aseptic meningitis, poliomyelitis-like paralysis, brainstem encephalitis, and neurogenic pulmonary edema (6, 36). The incidence of EV71 appears to have increased in the Asian Pacific region since 1997. Several large outbreaks of HFMD have occurred with high rates of morbidity and substantial numbers of deaths (5, 6, 8, 9, 11, 21, 25, 27–29, 36, 40, 42). An association between genogroup and severity of disease has not yet been reported (4, 6).

The increasing numbers of EV71 cases reported in Asia and the observation of an EV71 outbreak in The Netherlands in 2007 after a period of low endemicity of 21 years raised the question of whether the epidemiology of EV71 is changing. Therefore, we did a detailed analysis of the epidemiological and genetic data on EV71 circulation in The Netherlands over a 45-year period (1963 to 2008).

MATERIALS AND METHODS

Isolation of enteroviruses in The Netherlands. In The Netherlands only the severe, hospitalized cases of EV71 infection are diagnosed and reported as part of the national enterovirus surveillance system. Mild cases of EV71 infection are hardly reported since HFMD is not classified as a notifiable disease. Primary diagnosis for all enterovirus infections in The Netherlands is performed by virological laboratories that participate in the Weekly Sentinel Surveillance System of the Dutch Working Group on Clinical Virology. Fecal samples, throat swabs, and cerebrospinal fluid (CSF) samples are collected from children with systemic viral infection, varying from meningitis to gastrointestinal disorders. The clinical samples are cultured on combinations of enterovirus-sensitive cell lines: RD, tertiary monkey kidney, LLC-MK2, Vero, HEp-2, and various human fibroblast cell lines. Viral isolates with an enterovirus-characteristic cytopathic effect are confirmed as enteroviruses by an immunofluorescence test with broadly reactive monoclonal antibodies (Dako, CA) (41, 47) or by a specific PCR assay (32, 33). Typing of enterovirus-positive isolates is locally performed by the virological laboratories by the use of serum neutralization tests with polyclonal typing pools (provided by the National Institute for Public Health and the Environment, RIVM, Bilthoven, The Netherlands) (46). Cell cultures of nontyped and nontypeable isolates are routinely sent to the RIVM for typing and characterization and form the basis for the historical RIVM collection of EV71.

Review of historic collection at RIVM for representativeness. In order to determine if differences in the surveillance setup influenced the data and EV71 isolates collected, a review was done of the protocols and technical developments from the beginning of the surveillance data collection.

RNA extraction. Viral RNA was extracted from cell cultures using a MagNA Pure LC Total Nucleic Acid Isolation Kit with a MagNA Pure LC instrument (Roche Diagnostics, Almere, The Netherlands). Lysis was done by adding 100 μl of cell culture to 400 μl of lysis binding buffer (provided in the kit). Extraction was performed according to manufacturer's instructions. Viral RNA was eluted in 50 μl of elution buffer.

EV71 PCR assay. The VP1 gene was amplified as two overlapping regions using the 159F (5′-ACYATGAAAYTGTGCAAGG-3′), 162R (5′-CCRGTAG GKGTRCACGCRAC-3′), 161F (5′-CTGGGACATAGAYATAACWGG-3′), and NP1Anew (5′-CCACYCTGAAGTTGCCCACG-3′) primers, which, except

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for NP1Anew, have been published previously (4). The previously published NP1A primer did not align optimally with our EV71 reference strains.

Reverse transcriptions were performed to convert the viral RNA to cDNA. For this reaction, 5.0 μl of the isolated viral RNA was incubated at 42°C for 60 min together with 1.0 μl of 25 μM antisense primer, 3.0 μl of 10× PCR buffer (pH 8.3) (100 mM Tris-HCl, 500 mM KCl), 1.8 μl of 25 mM $MgCl_2$, 2.0 μl of 10 mM concentrations of the deoxynucleoside triphosphates, 1.2 μl of 10 U/μl avian myeloblastosis virus reverse transcriptase (Promega, Leiden, The Netherlands), 0.4 μl of RNase inhibitor (Amersham, Life Science), and 15.6 μl of H_2O. The reaction was finished by incubation of the samples at 95°C for 5 min, followed by incubation on ice.

In total, 15 μl of the cDNA was used for the PCR amplification, together with 3.5 μl of 10× PCR buffer, 1.8 μl of 25 mM $MgCl_2$, 1 μl of 25 μM forward primer, 0.4 μl of 5 U/μl *Taq* polymerase (Roche), and 28.3 μl of water. The PCR was carried out for 1 cycle of 3 min at 94°C and 30 cycles of 45 s at 94°C, 45 s at 42°C, and 1 min at 68°C, with a final cycle of 7 min at 68°C. Amplicon size analysis was performed using gel electrophoresis. Purification of the PCR products was performed according to the manufacturer's protocol (QIAquick PCR Purification Kit; Qiagen).

Sequencing of the PCR products was carried out with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.2 (Applied Biosystems, Foster City, CA) on an automated sequencer (Applied Biosystems model 3700) using the 159F, 162seq (5′-GTTATRTCTATRTCCCAGTT-3′), 161F, and NP1Anew primers.

Phylogenetic analysis. Editing of the sequence data was performed using Bionumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Isolates were assigned to specific genogroups and subgenogroups according to previously defined nomenclature (4). Phylogenetic dendrograms were generated by the neighbor-joining method (bootstrap analysis with 1,000 pseudoreplicate data sets) using TREECON software, version 1.3b (43). The reference strains used in the phylogenetic analysis are presented in Table 1.

Comparison with globally available data from systematic literature review. Data on the global presence of EV71 subgenogroups were derived by an extensive literature review using the Pubmed and Scopus literature databases.

During the first search, abstracts and/or their titles were screened for the terms "enterovirus 71," "enterovirus type 71," "hev71," "hev-71," "ev71," or "ev 71." During the second search articles were screened on the basis of their main keywords: "outbreaks," "genotype," or "phenotype." Alternatively, articles were screened on basis of their scope: "epidemiology," "classification," "genetics," "pathogenicity." A third search was performed during which titles were screened for the terms "outbreak*," "epidem*," "case*," "fatal," "evolution," "genotyp*" (title/abstract), "genogroup*" (title/abstract), "subgenogroup*" (title/abstract), "phenotyp*," "phylogen*," "typing," "types," "serotyp*," "identificat*," "characteri*," "genetic," "circulation," or "new". Articles that were selected on the basis of the first search in combination with the second search or third search were used for reviewing. Year and country of detection and subgenogroup were recorded and used for a comparative analysis with the epidemiology in The Netherlands.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB491212 to AB491220.

TABLE 1. Reference strains used for phylogenetic analysis

Reference strain	Year of isolation	Place of isolation	Genogroup	Accession no.
BrCr	1970	United States	A	AB433863
2608-AUS-74	1974	Australia	B1	AF135885
2609-AUS-74	1974	Australia	B1	AF135886
2230-NY-76	1976	United States	B1	AF135869
2258-CA-USA-79	1979	United States	B1	AF135880
2952-SD-81	1981	United States	B2	AF135888
3663-MA-82	1982	United States	B2	AF135889
6887-SYD-86	1986	Australia	C1	AY722887
2219-IA-87	1987	United States	B2	AF009539
7238-AK-USA-87	1987	United States	C1	AF135952
7628-PA-USA-89	1987	United States	B2	AF009530
2222-IA-USA-88	1988	United States	B2	AF009540
Y90-3205	1990	Japan	B2	AB433863
2640-AUS-95	1995	Australia	C1	AF135946
2641-AUS-95	1995	Australia	C2	AF135947
2355-OK-97	1997	United States	C2	AF135942
MY821-3-SAR-97	1997	Sarawak	B3	AY125997
2896-TAI-98	1998	Taiwan	B4	AF286516
4350-SIN-98	1998	Singapore	B3	AF376119
TW-2086-98	1998	Taiwan	C2	AF116819
SHZH-CHN-98	1998	China	C4	AY465356
26 M-AUS-2-99	1999	Australia	B3	AF376101
F1-CHN-00	2000	China	C4	AB115490
H25-CHN-00	2000	China	C4	AB115492
IM/AUS/12/00	2000	Australia	C1	AF376098
S2861-SAR-00	2000	Sarawak	B4	AF376085
2027-SIN-01	2001	Singapore	B4	AF376111
01-KOR-00	2003	Korea	C3	AY125966
03-KOR-00	2003	Korea	C3	AY125968
TW-2004-104	2004	Taiwan	C4	DQ666684
1301V/VNM/05	2005	Vietnam	C5	AM490149
2928-Yamagata-06	2006	Japan	C4	AB433878
H0/6364/255/2006	2006	United Kingdom	C2	AM939607
EV71/BRU/2006/33930	2006	Brunei	B5	FM201328
1961-Yamagata-07	2007	Japan	C4	AB433892

RESULTS

Isolation of EV71. A review of the protocols and technical developments showed that diagnosis of EV71 has consistently been performed since 1963. Cell lines susceptible to EV71 and standardized serological typing reagents have been used throughout the years (24). EV71 cannot be neutralized by these reagents and formed part of the untypeable cell cultures that were routinely submitted to the RIVM for typing during the entire study period. Monospecific typing sera against EV71 became available at the RIVM in 1977, and isolates that could not be typed in the years 1963 to 1976 were tested retrospectively. A representative selection of the isolates was included in the RIVM biobank.

Trends in EV71 reporting. Between 1965 and 2008, 346 EV71 isolates were identified (Fig. 1). In total, 198 of these isolates (57%) from the same number of patients and 1 EV71 isolate from 1963 were stored and used in this study. Data on the origin of clinical samples, date of isolation, clinical presentation, and age of patient were obtained from the submission forms sent by the virological labs. In total, 55.3% of the EV71 isolates were obtained from feces; 15.1% were from throat swabs; 0.5% were from nose swabs; 3% were from fluid from vesicles on hands, feet, and mouth; and 0.5% were from CSF. For 25.6% of the isolates collected between 1982 and 2007, the sample origin was not known. In 1986 and in 2007 an increase in the number of cases of EV71 infection requiring hospitalization was reported. A mean EV71 isolation rate of 4.2% in 1986 and 6.3% in 2007 versus 0.5% in the preceding and following years indicates that these peaks are real.

Change of subgenogroup over time. The complete VP1 regions of 199 EV71 strains isolated in The Netherlands in the years 1963 to 2008 were sequenced successfully and were assigned to specific genogroups and subgenogroups on the basis of robust phylogenetic clustering with EV71 reference strains from the GenBank (4). Figure 2 shows the genetic relationships of Dutch isolates representative for the collection studied and the reference strains obtained from GenBank.

In total, 85 isolates clustered with reference strains of genogroup B, of which 28 isolates belonged to subgenogroup B1, and 47 isolates belonged to subgenogroup B2. The remaining 10 isolates from 1963 to 1967 formed a distinct cluster within genogroup B, with 90.51% homology to subgenogroups B1 and

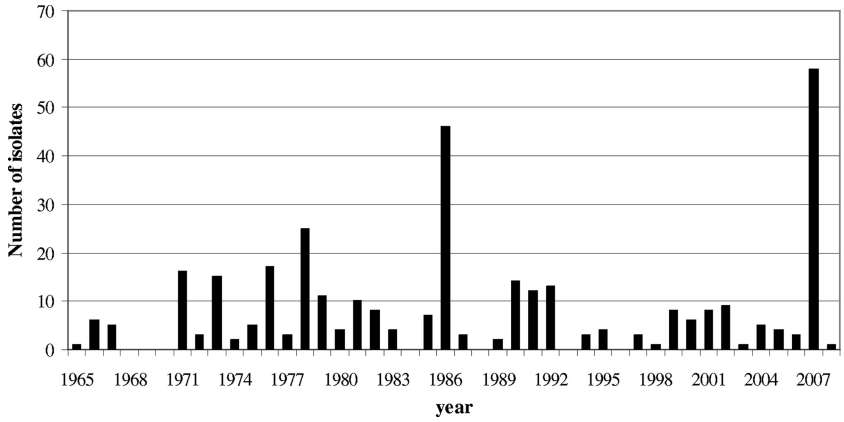

FIG. 1. Number of EV71 cases reported by members of the Weekly Sentinel Surveillance system of the Dutch Working group on Clinical Virology (1965 to 2008).

B2. This difference justifies the classification of these isolates in a new subgenogroup, B0. A clear change in the dominating subgenotype over time could be observed when the genotyping results were added to the surveillance data (Fig. 3). Isolates of subgenogroup B0 were observed in 1963 to 1967, followed by a predominance of strains belonging to subgenogroup B1 from 1971 to 1979. In 1977, strains of subgenotype B2 were observed for the first time in The Netherlands at a low frequency (2 in

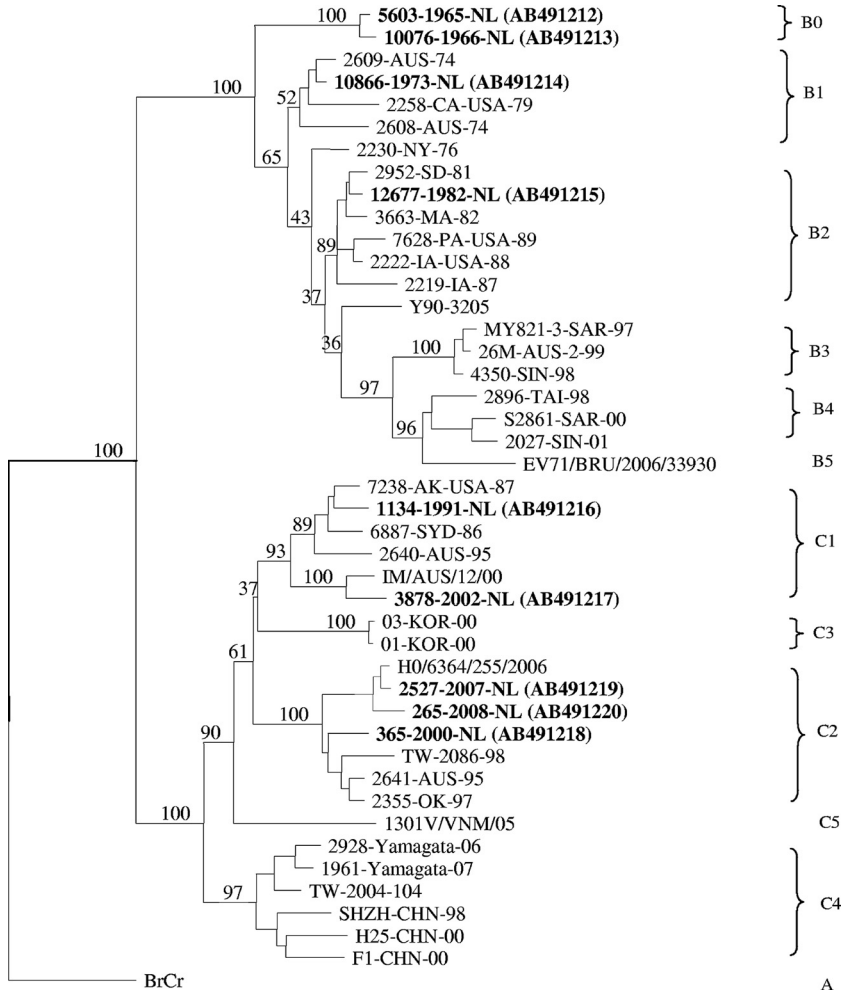

FIG. 2. Phylogenetic analysis of EV71 isolates from the Netherlands (bold) and reference strains. The dendrogram was generated by the neighbor-joining method (bootstrap analysis with 1,000 pseudoreplicate data sets) on the basis of a multiple alignment of the nucleotide sequences of the VP1 region (891 nucleotides).

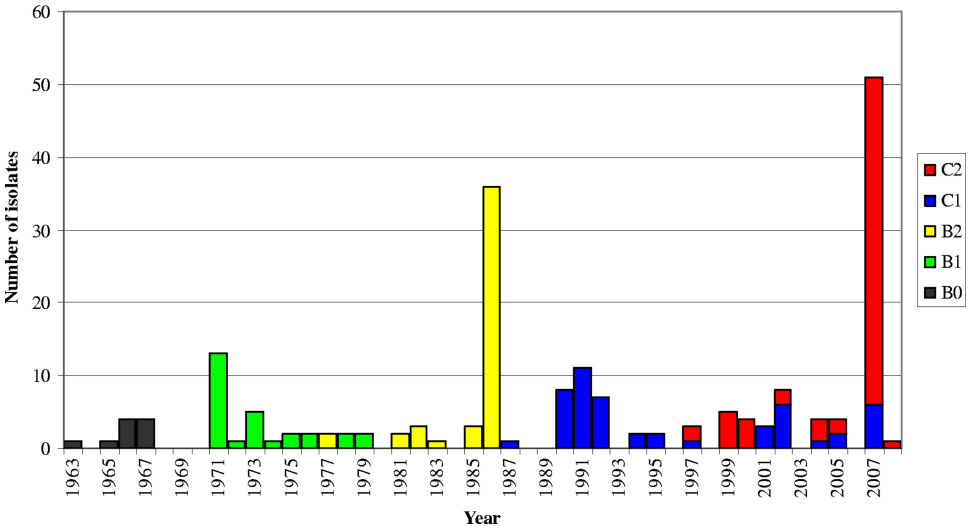

FIG. 3. Successive appearance of EV71 subgenogroups in The Netherlands from 1963 to 2008.

total), and B2 was the only subgenogroup observed in The Netherlands from 1981 to 1986, with a peak of hospitalized cases in 1986. No isolates were found in this study that clustered within subgenogroups B3 and B4.

After 1986, no strains of genogroup B were observed. All Dutch EV71 strains (114 in total) isolated after 1986 were assigned to genogroup C. Fifty isolates belonged to subgenogroup C1 (isolated in 1987 to 1997 and 2000 to 2007), and 64 belonged to subgenogroup C2 (dominating in 1997 to 2000 and 2002 to 2008). Only one EV71 strain (subgenogroup C2) was isolated in 2008. In this study no strains were observed that clustered within the C3, C4, or C5 subgenogroups.

Seasonal distribution of EV71. The month of sampling was known for 168 EV71 isolates (82%). Cases of EV71 infection were reported throughout the year, with a peak in the summer months of June and July. A second, but smaller, peak was observed in September and October (Fig. 4), including subgenogroups of both genogroup B and C. No difference in seasonal distribution could be observed between isolates belonging to genogroups B and C.

Clinical data. Clinical data were known for 78 children infected with EV71 of genogroup B (92%) and 62 children infected with EV71 of genogroup C (54%) (Table 2). In both genogroups the major reported clinical manifestations were fever, meningitis or encephalitis, and gastrointestinal symptoms (diarrhea and vomiting). The number of cases of typical HFMD (vesicles on hand, feet, and in mouth) was low, as expected, since only hospitalized cases of EV71 infection are usually diagnosed and reported in The Netherlands. There was no indication of EV71-related fatalities for the patients of this study.

The majority of the children showed more than one clinical symptom. For statistical analysis, the children were therefore categorized to one of the following groups: children with neu-

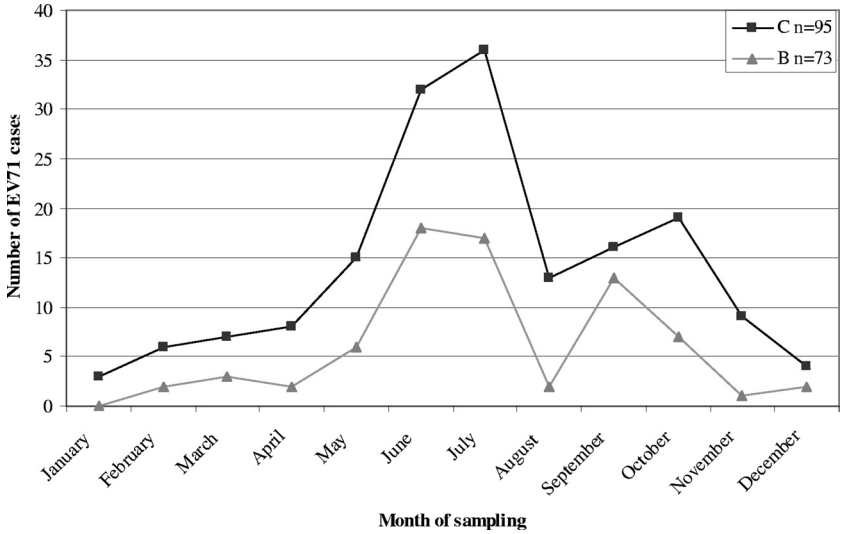

FIG. 4. Seasonal distribution of Dutch EV71 isolates, 1963 to 2008.

TABLE 2. Clinical symptoms of children infected with EV71[a]

Symptom	No. of instances (%) by EV71 subgenogroup		*P* value[b]
	B	C	
Fever	31 (40)	31 (50)	0.236
Gastrointestinal symptoms	11 (14)	21 (34)	0.008
Meningitis/encephalitis	50 (64)	11 (18)	0.000
Paresis	2	0	0.503
Convulsions	3	2	1.000
Headache	2	1	1.000
Erythema	1	0	1.000
Vesicles hand, feet, mouth	7	6	1.000
Exanthema	2	4	0.406
Laryngitis	0	1	0.406
Stomatitis	3	2	1.000
Inflam. upper resp. tract/otitis	9	3	0.227
Parotitis	2	0	0.503
Pneumonia	0	1	0.443
Bronchitis	4	2	0.963

[a] The total number of children infected was 78 for subgenogroup B and 62 for subgenogroup B. The number of children with neurological complications was 55 (71%) and 13 (21%), respectively; the number with other complications was 23 (29%) and 49 (79%) for infection with subgroups B and C viruses, respectively.
[b] *P* values were calculated with a two-tailed Fisher's test on the basis of number of children for whom clinical data were available.

rological complications (like meningitis, encephalitis, or convulsion) or children with other, milder complications only (i.e., gastrointestinal symptoms, rash, inflammation of the upper respiratory tract, or HFMD) (Table 2). Neurological complications were significantly more often reported among children infected with EV71 of genogroup B than among children infected with genogroup C ($P = 0.000$; odds ratio, 9.57; 95% confidence interval, 4.13 to 22.63). Gastrointestinal disease appeared to be more associated with genogroup C infection ($P = 0.008$; odds ratio, 3.12; 95% confidence interval, 1.27 to 7.89).

The age of children with EV71 infection was known for 166 children (genogroup B, $n = 72$; genogroup C, $n = 94$) (Fig. 5). The age of children infected with EV71 of genogroup B (median of 24 months) was overall significantly higher than the age of children infected with EV71 of genogroup C (median of 5 months) ($P = 0.000$, one-way analysis of variance). Comparison of the ages of children with neurological complications ($n = 62$; median of 24 months) and children with other, milder symptoms ($n = 62$; median of 13 months) showed that there was no significant difference in age ($P = 0.539$, one-way analysis of variance), excluding a correlation between the high number of neurological cases among children infected with genogroup B and the significantly higher ages of these children.

Comparison with globally available data from systematic literature review. Data on the global presence of EV71 subgenogroups were derived by an extensive literature review. In total, 326 articles were selected on the basis of our search strategy, and of these 30 papers contained data meeting our criteria. These were finally used to record place and time of isolation and subgenogroup (Fig. 6).

Except for some cases of genogroup B2 infection in Japan and Taiwan, no data were reported on EV71 infections in the Asian region before 1997. Reports on EV71 epidemiology outside of this region showed circulation of B1 and B2 strains in Europe, the United States, and Australia in the 1970s up to 1986/1987. In Japan subgenogroup B2 was observed until 1989. The shift of genogroup B to genogroup C infection observed in our country in 1987, followed by the prevalence of genogroup C1 and, later, C2, also occurred in the United States and Australia in the same period of time. Recent reports on EV71 circulation in the United Kingdom, Austria, and Norway confirmed analogous circulation of C1 and C2 strains. Strains of subgenogroups B3, B4, C3, C4, and C5 have caused large outbreaks in the Asian Pacific region since 1997. Except for two infections with subgenogroup C4 in Austria in 2004, these subgenogroups have not been observed outside the Asian Pacific region.

DISCUSSION

This study describes the epidemiology of EV71 in The Netherlands from 1963 to 2008. Consistency in typing of EV71 since 1977 and extensive retrospective testing of untypeable isolates

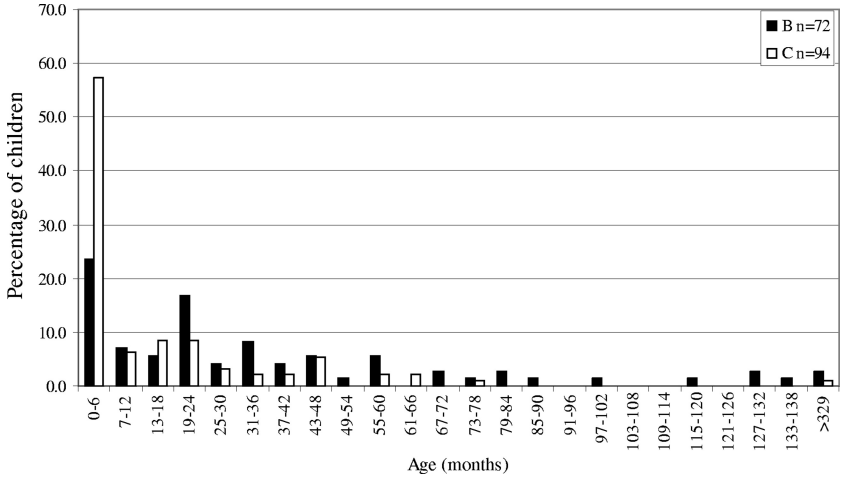

FIG. 5. Age of Dutch children infected with EV71, 1963 to 2008.

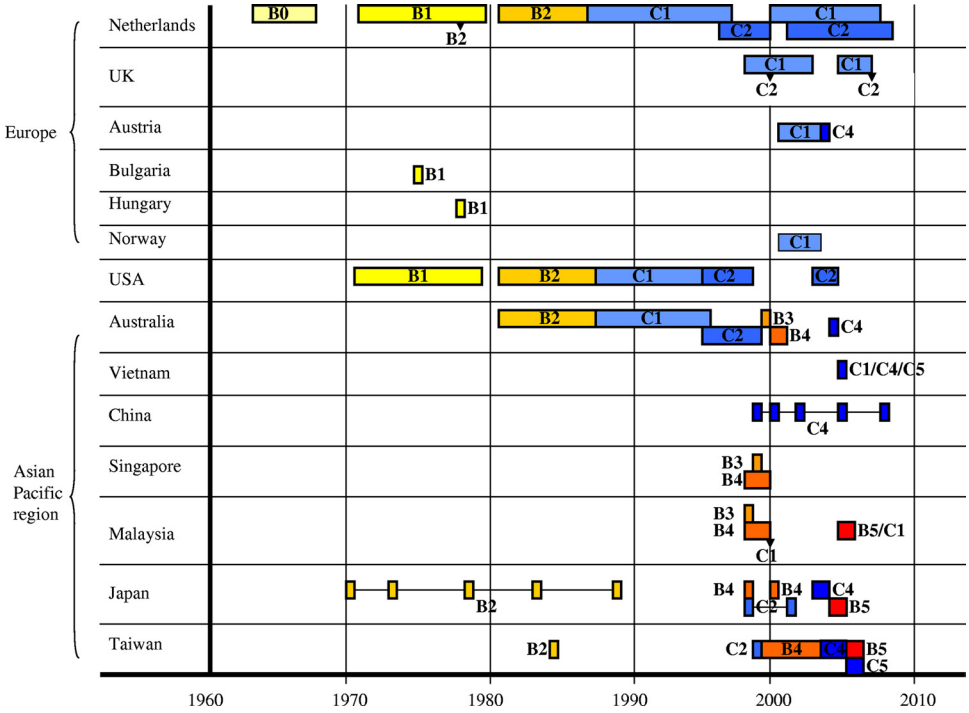

FIG. 6. Global presence of subgenogroups of EV71. Overview was made on the basis of an extensive literature review for the United Kingdom (2), Austria (35), Bulgaria (14), Hungary (30), Norway (45), the United States (3, 4, 36), Australia (4, 37, 39), Vietnam (42), China (11, 17, 18, 26), Singapore (6, 8, 29, 30, 40), Malaysia (1, 3, 5, 6, 13, 19, 29, 34, 36), Japan (3, 12, 31), and Taiwan (12, 22, 23, 25, 44). Black triangles represent single cases of subgenogroups other than the dominating subgenogroup in the period of time concerned.

from 1963 onwards made it possible to generate a detailed and realistic overview of a period of 45 years. This enabled observation of a clear change of prevailing subgenogroups over time in The Netherlands. The new subgenogroup B0, which was detected during the 1960s, has not been described before as retrospective typing of EV71 strains isolated before the first description of the EV71 serotype in 1974 has not been done previously. The seasonal distribution of EV71—a peak in the summer months of June and July and a second, but smaller, peak in September and October—does not differ from that of all other enterovirus infections diagnosed in The Netherlands (24).

The majority of the EV71 strains included in this study were isolated from feces and throat swabs. Only one isolate (0.5%) was obtained from CSF. This is in line with the results of a previous study in which specimens from the gastrointestinal and respiratory tracts were shown to have higher diagnostic yields for EV71 than CSF (36). Primary diagnosis of enteroviruses in CSF only from patients with neurologic disease results in an underestimation of the real number of EV71 infections.

Comparison of data on EV71 epidemiology in the public domain showed that the change in the prevailing subgenogroups (B1, B2, C1, and C2) and the shift of genogroup B to genogroup C infection occurred more or less simultaneously in Europe, the United States, and Australia. Since the first publications on EV71 in Asia in 1997, in addition to the strains found elsewhere, additional subgenogroups (B3, B4, C4, and C5) have been reported to cause large outbreaks with high rates of severe morbidity and mortality in this region only. The global nature of the epidemiology of some subgenogroups, however, raises the question of whether strains of the C4 and C5 lineages will also spread outside Asia. The epidemic of subgenogroup B2 infection in our country in 1986 was followed by a switch to a new genogroup (genogroup C), possibly driven by herd immunity against genogroup B. Recurrence of the same scenario for viruses of genogroup C (global epidemiology of C1 and C2) might imply that epidemics of subgenogroup C4 infections in other parts of the world will be unlikely and that there might be a switch to a new genogroup.

A change in the clinical presentation of EV71 infection over time has been suggested previously (3, 34). This is the first time, however, that a clinical difference between EV71 lineages has been described showing more severe disease associated with the genogroup B strains. These results, however, should be interpreted with caution. Mild cases of infection with genogroup B might have been reported less frequently during the 1980s than infections with genogroup C in recent years, and the higher average age of people infected may have contributed to more severe illness. In our data set, however, there was no significant correlation between the high number of neurological cases among children infected with genogroup B and the significantly higher ages of these children. In addition, fatal and severe cases of EV71 infection have been reported to occur especially among children of <4 years of age, with an increase in the number of cases with decreasing age (7, 10, 16, 20). During the outbreak of subgenogroup C4 infection in China in 2008, 63.6% of the Chinese children infected were <2 years of age, and 39% were <1 year of age (11). More data on the age of children infected with EV71 of different subgeno-

groups of B and C should be analyzed to verify differences in the age groups affected.

It is not clear why the Asian Pacific region has experienced an increased incidence of EV71 infection since 1997. A change in clinical presentation could coincide with a change in the viral transmission pathway. A shift of neurological complications to gastrointestinal disease with vomiting and diarrhea, in combination with other epidemiological parameters like crowding, lack of sanitation, and climate, could enhance virus transmission and explain the high rates of morbidity during epidemics in the Asian Pacific region. An increased susceptibility of the population to EV71 infection could be another explanation for the increased incidence. A study in Taiwan showed that EV71 seroconversion rates between 1994 and 1997 (a year before the large outbreak in 1998) were significantly lower (3% to 4%) than the rates before 1994 (7% to 11%) (27). As a consequence, the size of the susceptible population increased, potentially passing a critical threshold. It is of interest to study host susceptibility among the Dutch population before and after the large outbreaks in 1986 and 2007.

To our knowledge this is the first extensive description of EV71 epidemiology in Europe. This study suggests that the change in the circulating virus from genogroup B to genogroup C coincided with a change in clinical presentation and in age group affected. The epidemiology of EV71 subgenogroups B1, B2, C1, and C2 appeared to have a global nature, which implies that a subgenogroup C4 outbreak, as was observed in China in 2008, can be expected to occur in other parts of the world as well. Repeat of the 1986 scenario in The Netherlands would imply that new cases of severe EV71 infection will occur only after the introduction somewhere in the world of a new genotype virus, e.g., subgenogroup D (15). In line with this scenario is the isolation of only one EV71 strain in The Netherlands in 2008 under surveillance regimen identical to that of the preceding years.

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